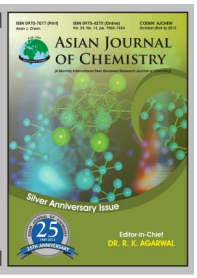
Asian Journal of Chemistry; Vol. 25, No. 15 (2013), 8579-8583

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.14847



Extraction and Determination of Chloramphenicol in Meat Using 1-Butyl-3-methylimidazolium Chloride-K_2HPO_4 Aqueous Two-Phase System Coupled with HPLC

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(*Received*: 21 November 2012; *Accepted*: 26 August 2013) AJC-14000

An ionic liquid-salt aqueous two-phase system (ILATPS) consisting of 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl) and K_2HPO_4, which is a simple, green and rapid sample pretreatment technique was used for the simultaneous separation, concentration and analysis of chloramphenicol (CAP) coupled with high-performance liquid chromatography. The influence factors on partitions of chloramphenicol were studied, including the types of ionic liquids and salts, the concentration of salt and chloramphenicol, temperature, pH and the volume of ionic liquid. In [C_4mim]Cl-K_2HPO_4 ILATPS, the extraction efficiency of chloramphenicol increased with the volume of [C_4mim]Cl increase and decreased with temperature increase. Under the optimal conditions, the average extraction efficiency of chloramphenicol in [C_4mim]Cl-K_2HPO_4 ILATPS was up to 95 %. This method has been satisfactorily applied to the analysis of chloramphenicol in meat samples with the detection limit of 0.23 ng g^{-1} and relative standard deviation (RSD) of 1.27 % (n = 7). The recovery of chloramphenicol was 94.4-107 % from meat samples with a RSD of 0.38-1.47 %, showing that this method was satisfactory for the separation of antibiotics.

Key Words: Ionic liquid, Aqueous two-phase system, Chloramphenicol, HPLC, Extraction.

INTRODUCTION

Chloramphenicol (CAP) which was first isolated from the bacterium Streptomyces venezuelae is active against vast gram-positive and gram-negative bacteria[1] in both humans and animals[2]. However, because chloramphenicol is often associated with harmful side effects in human, such as bone marrow depression and fatal aplastic anaemia[3], it has been prohibited from application to food production in the European Union[4]. Nevertheless, due to its easy access and low cost, chloramphenicol is still illegally used in animal farming. The methods for the determination of chloramphenicol include microbial assay[5], enzyme-linked immunosorbent assay (ELISA)[6,7], fluorometric screening method[8], sensors[9] and chromatographic methods using gas chromatography (GC)[10] and liquid chromatography (LC)[11,12]. However, these methods are high cost, short of the necessary sensitivity or time-consuming that requires complex procedures to prepare the sample. Furthermore, because of complexity and low concentration of chloramphenicol residues in environment, we require a rapid and effective sample pretreatment method prior to quantitative analysis of trace level of chloramphenicol. At present, solid-phase extraction (SPE)[7,10] and liquid-liquid extraction (LLE)[13,14] are the mainly pretreatment methods for the determination of chloramphenicol. However, solid-phase extraction requires a solvent desorption step which is time-consuming, complicated and generic sorbents usually lack selectivity; while traditional liquid-liquid extraction usually requires poisonous and volatile organic solvents. Therefore, a simple, rapid and sensitive method is required for sample pretreatment and chloramphenicol analysis.

Aqueous two-phase extraction (ATPE) is one of liquid-liquid extraction technique, but different from traditional liquid-liquid extraction. Aqueous two-phase system (ATPS) is considered to be environmentally friendly due to the both phases consist of water and no use of volatile organic solvent in the whole process. Aqueous two-phase systems are formed when two mutually incompatible water-soluble polymers or one polymer and one salt are dissolved in water above a critical concentration. Aqueous two-phase system has been successfully applied in separation and purification of many biological materials such as proteins[15], nucleic acids[16] and other biological products[17]. To improve the extraction efficiencies and to minimize environmental impacts the replacement of ordinary organic solvents by ionic liquids (ILs) has been a promising alternative.

Recently, room temperature ionic liquids have received extensive attention because of their advantageous features such as negligible volatility, excellent chemical and thermal stability, nonflammability and good solubility[18]. Some ionic liquids can form aqueous two-phase systems when in contact with concentrated salts solutions. Ionic liquid-salt aqueous two-phase systems (ILATPSs) reported by Rogers *et al.*[19]. This techniques has many advantages, such as low viscosity, little emulsion formation, quick phase separation, no need of using volatile organic solvents, high extraction efficiency and gentle biocompatible environment[20]. Ionic liquid-salt aqueous two-phase systems have been successfully used in the separation, concentration and purification of proteins[21,22], metal ions[23] and antibiotics[24,25].

In this study, the ILATPS based on 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl) and K_2HPO_4, coupled with HPLC was applied to extract and determine chloramphenicol. The parameters influencing the partitions of chloramphenicol, such as the types of ionic liquids and salts, the concentration of salt and chloramphenicol, temperature, pH and the volume of ionic liquid, were discussed in detail. Under the optimal conditions, this method has been successfully used to the analysis of trace chloramphenicol in meat samples.

EXPERIMENTAL

The standard drug sample of chloramphenicol was procured from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 1-Butyl-3-methylimidazolium chloride ([C_4mim]Cl), 1-hexyl-3-methylimidazolium chloride ([C_6mim]Cl) and 1-benzyl-3-methylimidazolium chloride ([C_7H_7mim]Cl) with a mass fraction purity of greater than 0.99 were obtained from Chengjie Chemical Co., Ltd. (Shanghai,China). Methanol of HPLC grade was from Sinopham Chemical Reagent Co., Ltd. (Shanghai, China). All chemicals were of analytical grade and all solutions were prepared using deionized water. The stock solution of chloramphenicol was prepared by dissolving in methanol at a concentration of 500 μg mL^{-1} and should be replaced every 2 months. Each working standard solution of chloramphenicol was prepared by appropriate dilution of the stock solution using deionized water. All solutions were stored at 4 ºC.

The BS124S electron balance (Beijing Sartorius instrument Co., Ltd., Beijing, China) was used for weighting. The pH was measured by a digital pH meter (Shanghai, China). The Anke centrifuge (Shanghai, China) was used to centrifuge. The temperature was controlled by a thermostatic water bath (Henan, China). An Agilent 1200 HPLC (Agilent, USA) equipped with a quaternary pump and an ultraviolet-visible detector was used for analysis of extraction products. The instrument control and data processing were actualized by using Agilent ChemStation software.

Preparation of actual samples: Fresh shrimp was purchased from the fish market of Zhenjiang, China. Shrimp was brought to the laboratory in iced condition and washed in water to remove the head, chitinous shells and body appendages. Then shrimp was peeled and deveined manually by wearing gloves. After that, the treated shrimp and the beef, chicken, pork purchased from local retail market were stored at -10 ºC and thawed several hours at ambient temperature before using. The trichloroacetic acid solution (10 mL, 15 % in water) containing different concentrations of chloramphenicol (0-120 ng mL^{-1}) was mixed with certain amount of minced samples and then the mixture was thoroughly mixed using a homogenizer-disperser, centrifuged at 3,000 rpm for 0.5 h. Finally the supernatant was filtered through microfilter with a pore size of 0.45 μm to remove the denatured proteins. The extracts were stored at 4 ºC for further use.

General procedure: In a 10 mL centrifugal tube, 9 mL, 0.55 g mL^{-1} of K_2HPO_4 solution containing 2 μg mL^{-1} of chloramphenicol was added and then added 1 mL of [C_4mim]Cl (80 %, mass fraction). The mixture was gently stirred for 5 min at room temperature and then centrifuged at 2,000 rpm for 0.5 h. After centrifugation, the centrifuge tubes were placed into a thermostatic waterbath at 25 ± 0.05 ºC for 2 h to equilibrate and allow for phase separation. The volume of the top and bottom phases was recorded precisely. The desired pH was adjusted by hydrochloric acid or ammonia water if necessary. Chloramphenicol in the top phase was determined by HPLC after extraction without any treatment. An analytical reversed-phase column was used for chromatographic separations at the column temperature of 25 ºC. The ratio of mobile phase of methanol and water was 43:57 at the flow rate of 1.0 mL min^{-1}. The injected volume was 20 μL and the column effluent was monitored at a wavelength of 278 nm.

Partition parameters of chloramphenicol: Extraction efficiency of chloramphenicol can be calculated by

$$E = \frac{C_t V_t}{m_s}$$

where C_t represented the equilibrium concentration of chloramphenicol in the top phase, V_t was the volume of the top phase after phase separation and m_s was the mass of chloramphenicol initially added.

RESULTS AND DISCUSSION

Effect of ionic liquids: In order to evaluate the ionic liquids species influence in the extraction of chloramphenicol several combinations were performed. The imidazolium-based ionic liquids, [C_4mim]Cl, [C_6mim]Cl and [C_7H_7mim]Cl were used to form aqueous two-phase systems with K_2HPO_4. The selected ionic liquids with different concentrations can form aqueous two-phase systems when the concentration of K_2HPO_4 was high enough. Three concentrations of ionic liquids (40, 60, 80 %, mass fraction) were chosen to discuss. From results, the extraction efficiency of chloramphenicol increased with the concentrations of ionic liquids increasing, because chloramphenicol was more inclined to concentrate in ionic liquid-phase and the mass of ionic liquid in ionic liquid-phase was increased with the increase of ionic liquid concentration. In Fig. 1, the extraction efficiency of chloramphenicol in different ionic liquid-K_2HPO_4 aqueous two-phase systems was shown when the concentration of ionic liquids was 80 %. At the same concentration of K_2HPO_4, the ability of ILATPSs for chloramphenicol extraction was different. When the concentration of K_2HPO_4 was in 0.55-0.75 g mL^{-1}, the extraction efficiency of chloramphenicol was relatively high in the three ILATPSs and

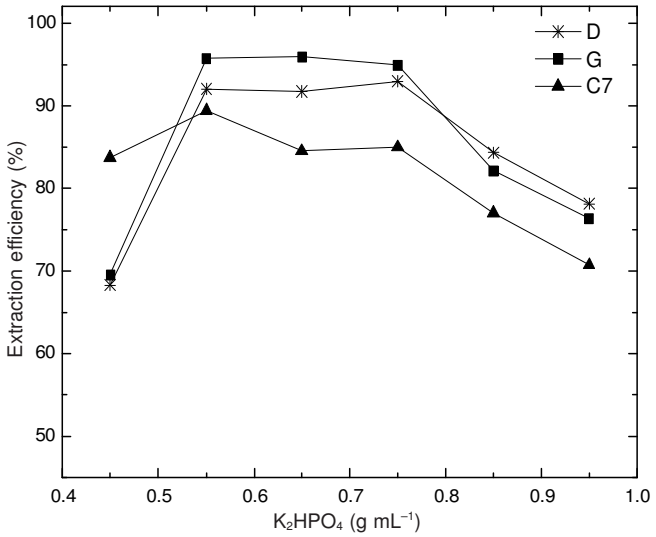

Fig. 1. Extraction efficiency (E %) of chloramphenicol in ionic liquid-K_2HPO_4 aqueous two-phase systems: ■, [C_4mim]Cl, 80 ; *, [C_6mim]Cl, 80 %; ▲, [C_7H_7mim]Cl, 80 %

the ability of ionic liquids for extraction followed the order [C_4mim]Cl > [C_6mim]Cl > [C_7H_7mim]Cl. So [C_4mim]Cl was chosen as extractant in subsequent experiments.

Effect of salts: Changing the type and concentration of salt is known to influence the partition behaviours of many biological materials. Hence, it is thought desirable to study the effects of salts on partition of chloramphenicol in [C_4mim]Cl-salt ILATPS. Various salts, acid, neutral and basic salts were used to form aqueous two-phase system with 80 % [C_4mim]Cl. The basic salts, K_3PO_4, K_2CO_3, $(NH_4)_2HPO_4$, Na_2CO_3 and K_2HPO_4 can form aqueous two-phase system with [C_4mim]Cl, while acid salts, KH_2PO_4 and $(NH_4)_2SO_4$ and neutral salts, KCl and Na_2SO_4 cannot cause phase separation with [C_4mim]Cl. In Fig. 2, there was no chloramphenicol extracted to ionic liquid-phase in [C_4mim]Cl-K_3PO_4 aqueous two-phase system, so the extraction efficiency of chloramphenicol was zero. In the other aqueous two-phase systems, chloramphenicol was extracted to the ionic liquid-phase and the maximum extraction efficiency higher than 95 % was in [C_4mim]Cl-K_2HPO_4 ILATPS. In subsequent experiments, the factors influencing chloramphenicol partition were investigated in [C_4mim]Cl-K_2HPO_4 ILATPS.

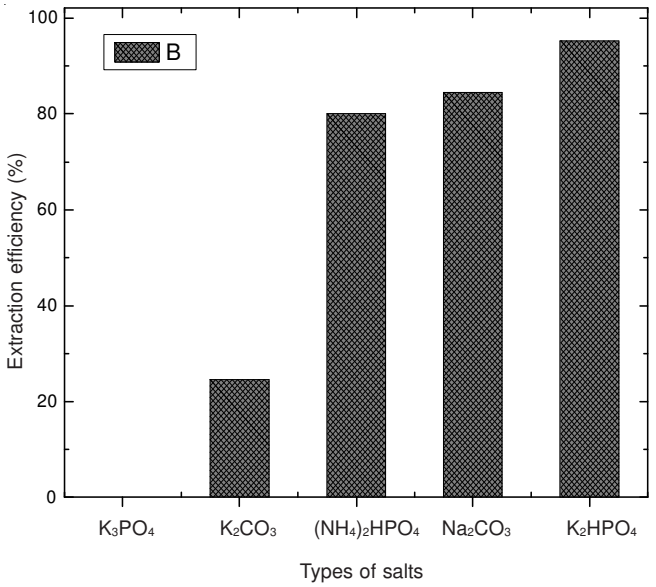

Fig. 2. Extraction efficiency (E, %) of chloramphenicol in [C_4mim]Cl-salt aqueous two-phase systems

Effect of K_2HPO_4 concentration: The influence of different concentrations of K_2HPO_4 on the extraction efficiency of chloramphenicol was discussed in detail in [C_4mim]Cl-K_2HPO_4 ILATPS. After complete phase separation, chloramphenicol was mainly transformed into the top phase. We discussed the interrelationship of extraction efficiency and salt concentrations in the range of 0.45-0.95 g mL^{-1} (Fig. 1). From Fig. 1, firstly, the extraction efficiency of chloramphenicol increased with the concentration of K_2HPO_4 from 0.45 to 0.55 g mL^{-1}; secondly, the extraction efficiency maintained at *ca.* 95 % with K_2HPO_4 concentration in 0.55-0.75 g mL^{-1}. This is because that the salting-out effect of K_2HPO_4 have reach to a maximum degree, it was not able to improve the extraction efficiency of chloramphenicol. Then decreased with the increase of K_2HPO_4 concentration, because excess salt compete for water molecules with chloramphenicol in the top phase so that part of chloramphenicol was transferred to the bottom phase and accordingly, the extraction efficiency decreased. The concentration of K_2HPO_4 used in [C_4mim]Cl-K_2HPO_4 ILATPS was 0.55 g mL^{-1}.

Effect of temperature: The effect of temperature on the extraction efficiency in [C_4mim]Cl-K_2HPO_4 ILATPS was investigated (Fig. 3). The extraction efficiency had only risen slightly when the temperature was from 15 to 25 ºC, then the extraction efficiency of chloramphenicol continuously reduced from 25 to 85 ºC. With the temperature rising, the solubility of K_2HPO_4 was improved and more [C_4mim]Cl dissolved into salt enriched bottom phase, resulting in the reduction of the salting-out effect of salt. Under these circumstances, the capability of phase separation of ILATPS was decreased with increasing temperature and the extraction efficiency of chloramphenicol also decreased. When the temperature was below 60 ºC, the extraction efficiency was higher than 80 %. When the temperature exceeded 60 ºC, chloramphenicol

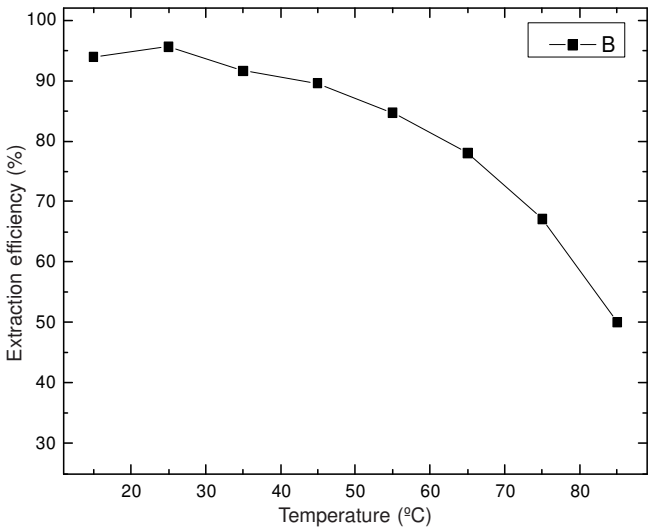

Fig. 3. Effect of temperature on extraction efficiency (E, %) of chloramphenicol in [C_4mim]Cl-K_2HPO_4 aqueous two-phase systems

would begin to decompose and the extraction efficiency fell drastically. Thus, only when the temperature remains below 60 ºC, this method provides a relatively wide temperature range for the study on the extraction behaviour of analytes. The whole experiment was carried out at 25 ºC.

Effect of pH: Experiments were performed by changing the pH from 7 to 12 by adding suitable hydrochloric acid or ammonia water. The chloramphenicol was stable in neutral and alkaline condition, while in acid or strong alkaline condition, chloramphenicol could not exist with the molecular solvent and it was decomposed. The extraction efficiency of chloramphenicol exceeded 90 % in pH 7.0-11.0, when pH reached 12.0, the extraction efficiency rapidly reduced to 70 %. The maximum extraction efficiency of chloramphenicol was 95 % in pH 10.0, the pH 10.0 can be selected as the optimal pH for chloramphenicol extraction. And the pH of K_2HPO_4 solution was 9.8, so it's not necessary to adjust the pH in the whole experiment.

Effect of the volume of [C_4mim]Cl: The influence of the amount of [C_4mim]Cl on the extraction efficiency of analytes was also investigated. With the increase of the volume of [C_4mim]Cl from 0.4 to 1.4 mL, the extraction efficiency of chloramphenicol continuously increased, there into, the extraction efficiency was higher than 90 % since the volume of [C_4mim]Cl was 0.8 mL. Generally, high extraction efficiency is crucial for the enrichment of trace component. In consideration of the cost, 1 mL [C_4mim]Cl was really appropriate for chloramphenicol extraction.

Effect of the concentration of chloramphenicol: The influence of the concentration of added chloramphenicol on the extraction efficiency was investigated in the range of 0.02-1.2 μg mL^{-1}. The extraction efficiency of chloramphenicol was 94.1-95.1 %. The extraction efficiency was almost unchanged with increasing the amount of added chloramphenicol. This indicates that extraction efficiency was insensitive to the concentration of chloramphenicol. In this study, 0.5 μg mL^{-1} was used as an appropriate concentration of chloramphenicol.

Method validation: The calibration curve was performed by adding standard chloramphenicol at seven different concentrations in the range of 10-230 ng mL^{-1} to [C_4mim]Cl-K_2HPO_4 ILATPS. After extraction, chloramphenicol concentration in the top phase was measured by HPLC. The obtained linear regression equation for chloramphenicol was Area = 0.13076629 × c – 0.2864981 with R^2 = 0.9995, where c was the concentration of chloramphenicol with the unit of ng mL^{-1}. The limit of detection (LOD) was 0.23 ng g^{-1} and the limit of quantification (LOQ) was 0.77 ng g^{-1}. The LOD was below the minimum required performance limit (MRPL) of 0.3 ng g^{-1} established by the European Commission[26]. To check the repeatability of the chromatographic procedure, analysis of standard chloramphenicol at a concentration of 1 μg mL^{-1} was performed (n = 7) and the relative standard deviation (RSD) was 1.27 %.

Sample analysis: The proposed extraction technique was applied to extract and determine chloramphenicol in meat samples. No contamination of chloramphenicol at detectable levels was found in meat samples before the chloramphenicol was added. After separation process, chloramphenicol in meat was extracted to the ionic liquid-phase and determined with the proposed HPLC method (Fig. 4 and Table-1). As shown in Table-1, the recovery was determined from spiked 0-120 ng mL^{-1} chloramphenicol. The recovery of chloramphenicol was 94.4-107 % with a RSD of 0.38-1.47 %, showing that the present extraction method has a satisfactory reproducibility and recovery for the determination of chloramphenicol. The present method can be gratifyingly applied to the quantitative analysis and determination of chloramphenicol in meat samples.

TABLE-1
ANALYSIS RESULTS (n = 3) FOR CAP IN MEAT SAMPLES

Samples	Concentration added (ng mL^{-1})	Concentration determined (ng mL^{-1})	Recovery (%)	RSD (%)
Chicken	0	ND[a]	–	–
	50	50.88	101.76	0.87
	70	72.80	104.00	0.61
	120	117.92	98.27	1.35
Beef	0	ND	–	–
	50	48.58	97.17	0.91
	70	70.51	100.72	0.63
	120	113.33	94.44	1.03
Shrimp	0	ND	–	–
	50	47.56	95.13	0.93
	70	74.58	106.55	0.59
	120	115.88	96.57	0.38
Pork	0	ND	–	–
	50	51.90	103.80	1.47
	70	70.76	101.09	0.62
	120	128.37	106.98	0.60

[a]Not found.

Conclusion

Ionic liquid aqueous two-phase system based on [C_4mim]Cl-K_2HPO_4 coupled with HPLC was a good method for the separation and concentration of trace chloramphenicol from meat samples. Compared with the traditional extraction method, the main advantages are simple operation, biocompatible environment, quick phase separation and high extraction efficiency. As a viable pretreatment technique, this extraction method, combined with HPLC, has been successfully applied to quantitatively determine chloramphenicol in meat samples. These results highlight new possibilities of ionic liquid-salt aqueous two-phase system (ILATPS) in the separation and purification of other small biomolecules.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (Nos. 21076098, 21206059 and 21207051), the Natural Science Foundation of Jiangsu Province (No. BK2010349 and BK2011529), China Postdoctoral Science Foundation funded project (No.2013M531284), Jiangsu Postdoctoral Science Foundation funded project (No.1202039C) and the Programs of Senior Talent Foundation of Jiangsu University (No.12JDG079).

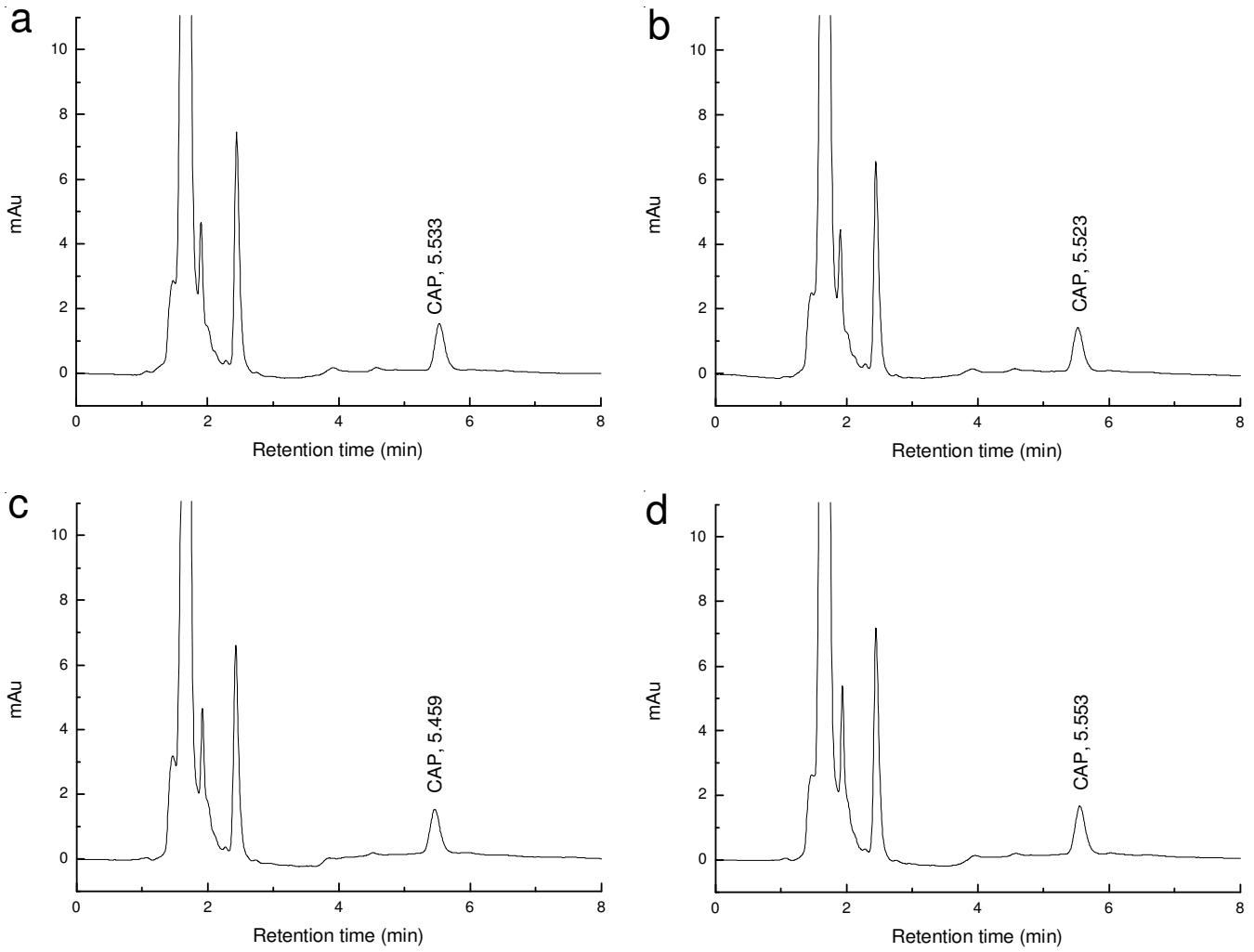

Fig. 4. HPLC chromatograms with UV detection after aqueous two-phase system extraction: (a) a sample of chicken added with 120 ng mL^{-1} chloramphenicol (b) a sample of beef added with 120 ng mL^{-1} chloramphenicol (c) a sample of shrimp added with 120 ng mL^{-1} (d) a sample of pork added with 120 ng mL^{-1} chloramphenicol

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